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Mosquito La protein binds to the 3′ untranslated region of the positive and negative polarity dengue virus RNAs and relocates to the cytoplasm of infected cells

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Abstract

The untranslated regions (UTRs) of the positive and negative strand RNAs of several viruses are major binding sites for cellular and viral proteins. Human La autoantigen is one of the cellular proteins that interacts with various positive strand RNA viral genomes including that of dengue virus (DEN) within the 5′- and 3′-UTRs of positive (+) and the 3′-UTR of negative strand (−) RNA, and with the nonstructural proteins NS3 and NS5, that form DEN replicase complex. Since DEN replicates in human and mosquito cells, some functional interactions have to be conserved in both hosts. In the present report, we demonstrate that mosquito La protein interacts with the 3′-UTRs of (+) and (−) polarity viral RNAs. The localization of La protein, examined by confocal microscopy, indicates that La protein is redistributed in DEN-infected cells. Furthermore, the presence of La protein in an *in vitro* replication system inhibited RNA synthesis in a dose-dependent manner, suggesting that La protein plays an important role in dengue virus replicative cycle.

Keywords: Replication; Dengue; UTR; La protein; Mosquito

Introduction

Dengue virus (DEN), a mosquito-borne virus of the *Flaviviridae* family, contains a single-stranded RNA (~11-kb) genome of positive polarity with a type I 5′-cap but no poly(A) tail. The viral RNA has a single long open reading frame (ORF) coding for a polyprotein which undergoes co- and post-translational processing into 10 mature proteins; three of these are structural proteins (envelope E, membrane M and capsid C) and seven are nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). In addition, the RNA genome contains two untranslated regions (UTR) flanking the ORF, and for DEN serotype 4 (DEN4), the 5′- and 3′-UTRs are 101 and 384 nucleotides (nt) long, respectively.

The UTRs of positive-strand RNA viruses are thought to serve various functions such as in initiation and regulation of viral translation, replication and assembly (Lindenbach and Rice, 2001; Proutski et al., 1999). The 3′-UTRs of mosquito-borne flaviviruses contain a conserved stem-loop (3′SL) structure within the last ~96nt (Brinton et al., 1986; Brinton and Dispoto, 1988; Grange et al., 1985; Mohan and Padmanabhan, 1991) and predicted pseudo-knot structures (Shi et al., 1996; Olsthoorn and Bol, 2001). There are two conserved sequences (5′- and 3′-CS1), one in the proximity of 3′ SL, and the other located just outside of the 5′-UTR. The 5′- and 3′-CS1 include the highly conserved motifs, known as cyclization sequences (CAUAUUGAC) that are complementary to each other (Hahn et al., 1987). The cyclization motifs are essential for negative (−) strand RNA synthesis in an in vitro assay that utilizes an exogenous subgenomic viral RNA template containing the 5′-and 3′ CS1 and either a membrane-bound viral

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replicase complex or a purified preparation of NS5 protein, the RNA-dependent RNA polymerase (RdRP) (Ackermann and Padmanabhan, 2001; You et al., 2001; You and Padmanabhan, 1999; Nomaguchi et al., 2004). Furthermore, the importance of cyclization motifs in viral replication was shown by using subgenomic replicon RNAs expressing reporters for several flaviviruses, Kunjin virus (Khromykh et al., 2001), West Nile virus (Lo et al., 2003), and Yellow fever virus (Corver et al., 2003; Jones et al., 2005) as well as using infectious RNAs (Alvarez et al., 2005b; Bredenbeek et al., 2003). These cyclization motifs as well as a newly identified motif upstream of the AUG codon (5′UAR) together with the complementary sequence located in the 3′-UTR (3′UAR) were shown to be required for cyclization of viral RNA by atomic force microscopy (Alvarez et al., 2005a). Partial or total deletion of different segments of the 3′-UTR which include the conserved sequence 2 (CS2), the repeated CS2 sequence (RCS2) reduces or abolishes viral replication without compromising translation. However, deletion, substitution, or point mutagenesis of the CS1, cyclization sequences, 5′ or 3′ UAR or the 3′SL sequences generates nonviable viruses (Alvarez et al., 2005b; Men et al., 1996; Zeng et al., 1998). These results strongly support that the conserved elements present within 5′- and 3′-UTRs play an important role for dengue virus replication (Alvarez et al., 2005b; You et al., 2001). In addition to the sequences required for cyclization, the requirement for secondary structural elements within both ends suggests that these regions may be involved in interaction with trans-acting factors to facilitate or regulate viral replication.

The RNA replication complex is assembled on cellular membranes (Mackenzie et al., 1998; Westaway et al., 1997, 1999; for a review, see Westaway et al., 2003). It consists of two hydrophobic viral proteins, NS2A and NS4A, an abundant luminal protein, NS1, and the proteins which have multiple enzyme activities such as the NS5 and NS3. The NS5 has a 2′-*O*-methyltransferase and RNA dependent RNA polymerase activities, and the NS3 has the serine protease catalytic domain, an NTPase/RNA helicase, and the 5′-RNA triphosphatase (for a review, see Lindenbach and Rice, 2003). Consistent with their putative role in viral replication, both NS3 and NS5 interact with the 3′-UTR of Japanese encephalitis virus RNA (Chen et al., 1997).

Besides the participation of viral proteins in replication, the possible role of cellular proteins in replication and translation processes has also been suggested. One such protein is the human La autoantigen which was shown to interact with both UTRs of genomic RNA and with the 3′-UTR of the negative strand RNA of DEN4 (De Nova-Ocampo et al., 2002; García-Montalvo et al., 2004; Yocupicio-Monroy et al., 2003). The human La protein also interacts with two components of the viral nonstructural proteins that form the DEN replicase complex, NS3 and NS5 (García-Montalvo et al., 2004). These results implicated a role for human La protein in viral replication. If interactions between a cellular component and viral factors are required for replication, it is then anticipated that such interactions are also conserved in different cell types that serve as hosts for viral replication. DEN can be grown in human, primate and mosquito cells. Thus, it is possible that the role of some cellular proteins that mediate translation and/or replication may be conserved in different hosts. Since DEN infection in humans requires first viral growth in mosquitoes, the study of the cellular and viral proteins required for translation and replication of DEN in mosquito cells, is essential to understand the replicative cycle of the virus and could give us important information about viral tropism and virulence.

In an aim to analyze the role of some proteins from mosquito cells in DEN replication, we employed different strategies in this study. First by mobility shift assays and UV-induced crosslinking, we demonstrate that the mosquito La protein interacts with sequences around nt 96 to 101 of the (−) strand RNA from DEN4 as well as with the 3′-UTR of (+) strand RNA. This interaction was corroborated using a purified recombinant mosquito La protein expressed in *E. coli*. The localization of the mosquito La protein, analyzed using polyclonal anti-La antibodies and confocal microscopy, indicates that La protein, which is predominantly localized in the nucleus of uninfected cells, is redistributed in the cytoplasm of DEN-infected cells. Furthermore, the presence of La protein in an in vitro replication system inhibited synthesis of (−) and (+) strand RNA from (+) and (-) subgenomic RNA template in a dose dependent manner, suggesting that La protein may play a regulatory role in DEN replicative cycle.

Results

C6/36 cellular proteins interact with DEN4 3′-UTR of (−) strand

In a previous study, we analyzed the ability of the 3′-UTR of DEN4 (+) strand RNA to interact with proteins present in uninfected and infected extracts from C6/36 cells (De Nova-Ocampo et al., 2002). In that study, we determined that the complete 3′-UTR (from nt 10260 to 10644) (Fig. 1) as well as the 3′SL (nt 10528 to 10644) interacted with eight proteins including the translation elongation factor EF-1α. Except for EF-1α, the identities of seven proteins in the mosquito cell extracts that interacted with the 3′-UTR of DEN4 were unknown. Based on our observations that the recombinant human La and PTB proteins (or La protein in human promonocytic U937 cell lysates) were able to bind to the 3′-UTR (+) (García-Montalvo et al., 2004), we examined whether mosquito La and PTB as well as PCBP2 could also be binding to the 3′-UTR using the antibodies directed against the respective proteins; however, these antibodies were unable to detect or detected poorly the proteins from mosquito cells. Moreover, we reported that La, calreticulin and PDI (protein disulfide isomerase) from the human cell line U937 interacted with the 3′-UTR from DEN4 (−) strand (Yocupicio-Monroy et al., 2003); however, it remained to be established whether the mosquito homologs could also interact with the 3′-UTR (−) strand. In this study, we sought to analyze and identify the uninfected or DEN-infected mosquito (C6/36) cell proteins present in S10 extracts that bound to a ^{32}P-labeled 3′-UTR of DEN4 (−) strand RNA in mobility shift assays. The RNA was derived from nt 101 to 1 of

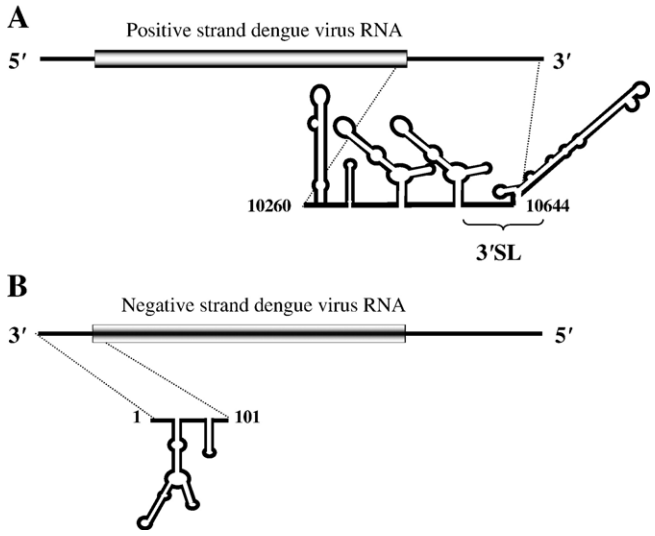

Fig. 1. Schematic representation of the 3′-UTR of the (+) and (−) strand RNAs. The complete 3′-UTR of the (+) strand RNA was obtained after in vitro transcription of a PCR fragment containing nt 10260 to 10644 from genomic RNA, while the 3′-UTR of the (−) strand RNA was obtained from a PCR fragment containing nt 101 to 1 (numbers correspond to the nt positions of the 5′-UTR of the (+) strand RNA) from the cDNA of the negative strand RNA.

(−) strand polarity (numbers correspond to the nt positions of the 5′-UTR of the (+) strand RNA) (Fig. 1B). The results indicated that labeled DEN4 3′-UTR of (−) strand formed four RNA–protein complexes with the S10 extract from uninfected (Fig. 2A, lane 2) and infected C6/36 cells (Fig. 2A, lane 6). The complex with the fastest migration, which was the most prominent, was referred to as CI, and the additional three minor complexes were designated as CII, CIII and CIV.

The stability of the RNA–protein complexes was analyzed in the presence of increasing KCl concentrations. The four RNA–protein complexes maintained their respective gel mobility up to 900 mM of KCl (Fig. 2A, lanes 3–5 and lanes 7–9 respectively), suggesting that the complexes were very stable.

The specificity of the interactions between the DEN4 3′-UTR (−) and cellular proteins from infected C6/36 extracts was determined in a competition experiment using 25-fold molar excess of homologous and heterologous unlabeled RNA fragments as competitors. The results indicated that the unlabeled homologous 3′-UTR (−) (nt 101 to 1) strongly reduced the formation of the four ribonucleoprotein complexes (Fig. 2B, lane 3). Although a similar amount of a heterologous RNA (nt 111 to 191 of Norwalk viral RNA) competed with formation of CIV and CII and partially with CIII (Fig. 2B, lane 4), it did not compete with CI, suggesting that CI is a specific complex. These data strongly suggest that the proteins present in the S10 extracts of infected C6/36 cells bound specifically to the 3′-UTR (−) RNA of DEN4.

To identify the proteins that bind to the 3′-UTR (−) of DEN4 virus, labeled RNA consisting of nt 101 to 1 was incubated with S10 extracts from uninfected and infected C6/36 cells, followed by UV-induced crosslinking. The 3′-UTR (−) RNA crosslinked with five major proteins with molecular weights of 80, 70, 62, 58, and a wide band of 51 kDa in both uninfected and infected S10 extracts (Fig. 2C, lanes 1 and 2 respectively). It is important to note that we could not detect any difference between the crosslinked proteins from uninfected and infected cell extracts similar to the observation with U937 cell extracts reported previously (Yocupicio-Monroy et al., 2003). In that study, we also demonstrated that the deletion of 6 nt from nt 101 to 96 (CCUUUU) (Fig. 2E), later referred as del-96, inhibited La protein binding. Therefore, to determine whether one of the proteins binding to the 3′-UTR (−) RNA could be the La protein, we used del-96 mutant as well as a second mutant called del-62 with a 6 nt deletion (from nt 62 to 68) (Fig. 2E) in which a stem present in the predicted secondary structure was eliminated without altering any protein binding. The del-62 mutant bound the same group of proteins as the wt 3′-UTR (−) RNA (Fig. 2D, lanes 1 and 2); however, the del-96 mutant exhibited a diminished crosslinking to the 51-kDa protein (Fig. 2D, lane 3). The failure of the del-96 mutant to interact with the 51-kDa protein suggests that this protein may possibly be the mosquito La protein.

La protein binds to the 3′-UTR (−) RNA

In an effort to determine if mosquito La protein is one of the proteins which interacts with the 3′-UTR, an immunoprecipitation assay was performed. After UV-induced crosslinking (Fig. 3A, lane 1), the crosslinked proteins were immunoprecipitated with human anti-La (Fig. 3A, lane 2) or anti-actin monoclonal antibody (Fig. 3A, lane 3). The human anti-La antibody was able to immunoprecipitate a faint radiolabeled protein of 50 kDa (Fig. 3A, lane 2) whereas the anti-actin antibody did not immunoprecipitate any radiolabeled protein (Fig. 3A, lane 3). The low amount of radiolabeled protein immunoprecipitated in these experiments can be explained by the reduced efficiency of anti-human La antibody to immunoprecipitate mosquito La protein; however, a sufficient amount of protein could be detected as seen in the autoradiograph strongly suggesting that mosquito La protein interacts directly with the complete 3′-UTR (−) of DEN4.

Further support for our observation that mosquito La protein interacts with the 3′-UTR (−) strand of DEN4 was obtained using the anti-mosquito La antibody in a supershift assay. The addition of the anti-La antibody to the binding reaction reduced the formation of the CI complex (Fig. 3B, lane 3), whereas the polyclonal anti-actin antibody did not have any effect (Fig. 3B, compare lanes 2 and 4), corroborating the binding of mosquito La protein to the 3′-UTR (−) strand. Using this approach, we also analyzed the binding of the 3′-UTR (+) strand RNA with the mosquito La protein. The results showed that the addition of the anti-La antibody interfered with the formation of the complexes formed with the 3′-UTR (+), especially two of the bands noticeably diminished as observed with the 3′-UTR (−) (Fig. 3C, lane 3), while the anti-actin antibody did not have any effect (Fig. 3C, compare lanes 2 and 4). In both cases, the anti-La antibody inhibited the interaction of the La protein present in the cell extract with the 3′-UTRs supporting the conclusion that

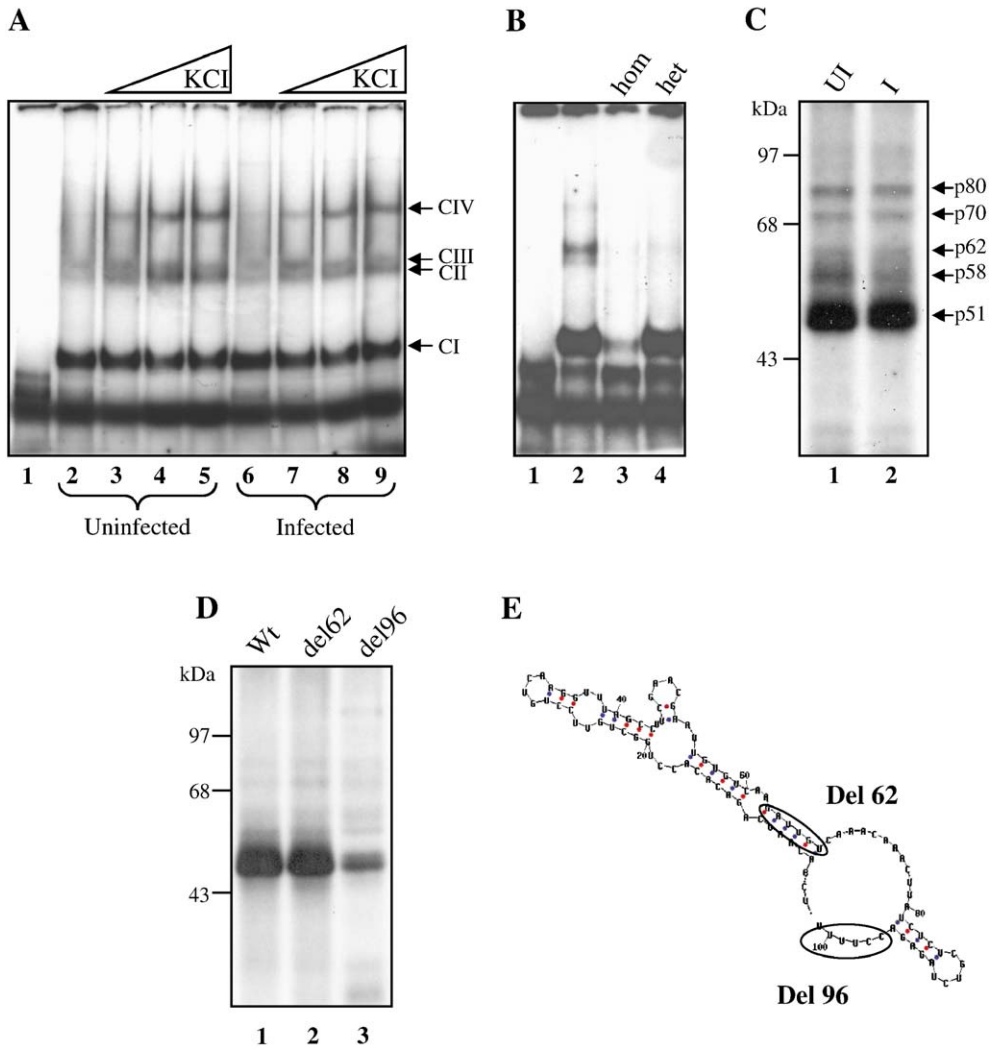

Fig. 2. Mobility shift and UV-crosslinking assay of the 3′-UTR (−) of DEN 4. (A) ^{32}P-labeled RNA consisting of the 3′-UTR (−) was incubated without (lane 1) or with 3 μg of S10 extract from uninfected (lanes 2 to 5) or infected (lanes 6 to 9) C6/36 cells in the presence of 300 mM (lanes 3 and 7), 600 mM (lanes 4 and 8) or 900 nM (lanes 5 and 9) of KCl. (B) Labeled 3′-UTR (−) was incubated without (lane 1) or with 3 μg of S10 extract from C6/36 infected cells (lanes 2 to 4) in the absence (lane 2) or in the presence of a 25-fold molar excess of unlabeled 3′-UTR (−) RNA (lane 3) or a 25-fold molar excess of unlabeled unrelated RNA, nt 111 to 191 of the Norwalk virus genome (lane 4). The complexes were assayed through a 6% native polyacrylamide gel. Arrows indicate the migration of the complexes. (C) Labeled 3′-UTR (−) was UV-crosslinked with uninfected (lane 1) or infected (lane 2) C6/36 cell extract. Proteins were separated by SDS-10% PAGE and detected by autoradiography. (D) Labeled 3′-UTR (-) consisting of nt 101 to 1 (lane 1), del-62 (lane 2) or del-96 (lane 3) were UV-crosslinked with 50 μg of infected S10 C6/36 cell extract. (E)The secondary structure predicted for the 3′-UTR (−) is presented. The 6 nt deletion produced in del-62 and in del-96 is shown. Molecular weight markers are indicated on the left. Arrows indicate crosslinked proteins with molecular masses given in kilodaltons.

mosquito La protein interacts with both 3′-UTRs of (+) and (−) strand RNAs.

Mosquito La protein expression

To analyze the ability of La protein to interact with the 3′-UTRs of DEN in more detail, the coding sequence of the mosquito La protein was cloned into the prokaryotic expression vector pProEX-HTc to obtain a recombinant plasmid pLaMos (5943 bp) as described in Material and methods section. The nucleotide sequence of mosquito La protein was previously reported (Pardigon and Strauss, 1996). The sequence comparison between mosquito and human La protein reported by Pardigon and Strauss indicated that they share considerable sequence identity in the N-terminal half, but not in the C-terminal domain, suggesting that probably some of the functions described for human La protein are probably not totally shared by the mosquito La protein.

The recombinant mosquito La protein with a hexahistidine tag was expressed according to the protocol provided by the manufacturer as described under Materials and methods section. Bacteria transformed with the vector pProEX-HTc or the pLaMos plasmid were grown in the presence or absence of IPTG for up to 5 h and the bacterial extracts were analyzed by SDS-10% PAGE. A 50-kDa protein was detected in the extracts obtained from the pLaMos-transformed bacteria compared to those from either uninduced or vector-transformed bacteria (data not shown). To demonstrate that the 50-kDa protein was

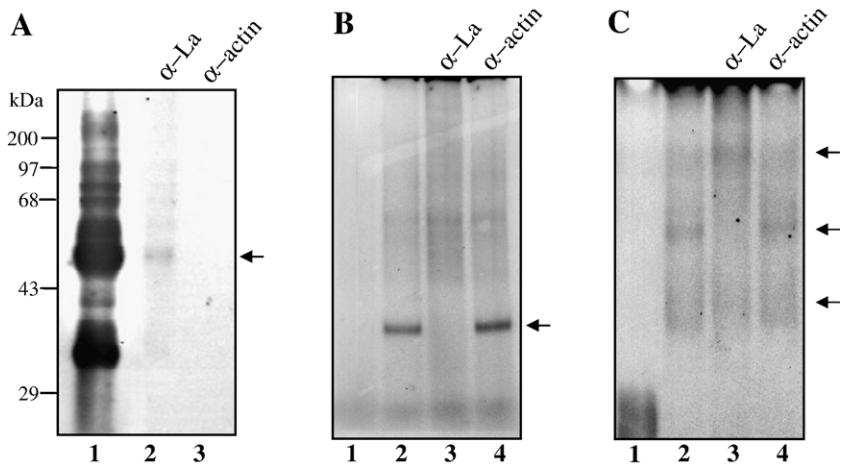

Fig. 3. Immunoprecipitation and mobility super shift assays performed with anti-human La protein antibodies. (A) Labeled 3′-UTR (−) was UV-crosslinked with 50 μg infected S10 C6/36 cell extract (lane 1). Crosslinked proteins were immunoprecipitated with anti-La (lane 2) or anti-actin (lane 3) monoclonal antibodies. Arrow indicates immunoprecipitated La protein. Molecular weight markers are indicated on the left. Labeled 3′-UTR (−) (B) or labeled 3′-UTR (+) (C) were incubated with infected extracts from C6/36 cells (lanes 2 to 4) in the absence (lane 2) or in the presence of anti-mosquito La (lane 3) or anti-actin (lane 4) antibodies. The complexes were resolved on a 6% native polyacrylamide gel. Arrows indicate the complexes altered by anti-La antibodies.

the recombinant His-tag mosquito La protein, a Western blot assay using polyclonal antibodies directed against His-tag was performed (data not shown). A major band of approximately 50 kDa was detected in the soluble fraction from bacteria transformed with the pLaMos plasmid at 1, 2, 3 and 5 h of induction, but absent in the soluble fraction from bacteria transformed with pProEX-HTc vector and in the extracts from noninduced bacteria (data not shown).

Mosquito La recombinant protein binds to DEN 3′-UTRs

The mosquito La protein present in total extracts (Fig. 4A, lane 1) was purified by passing through a Ni-NTA column, washed with buffer containing 10 and 20 mM of imidazole (Fig. 4A, lanes 2 and 3, respectively), and finally eluted with a buffer containing 250 mM imidazole (Fig. 4A, lane 4). The purified protein was dialyzed and concentrated with Amicon columns (Fig. 4A, lane 5). A minor band (43 kDa) observed in the eluted fraction was probably a breakdown product due to the high susceptibility of La protein to protease activity (Cardinali et al., 2003; Meerovitch et al., 1993).

Next, the ability of mosquito La protein to specifically interact with both 3′-UTRs of the (+) and (−) strand RNAs, was analyzed using 3 nM (Fig. 4B, lanes 1 and 4) or 6 nM (Fig. 4B, lanes 2 and 5) of the recombinant La protein or 6 nM of GST protein as a negative control (Fig. 4B, lanes 3 and 6). The results showed that the La protein bound to the labeled 3′-UTR (+) (Fig. 4B, lanes 1 and 2) and 3′-UTR (−) (Fig. 4B, lanes 4 and 5) RNA of DEN4. As expected, the GST did not bind to either RNA. From this result, we conclude that the mosquito La protein binds the 3′-UTR (+) and (−) strands RNAs.

La protein is redistributed during DEN infection

La protein is one of the cellular factors that contributes to the translation or replication of several viruses that replicate in the cytoplasm. This protein, under normal conditions, is located in the nucleus. Therefore, to play a role in translation or replication process in the virus life cycle in infected cells, the La protein is expected to be redistributed to the cytoplasm. This redistribution occurs in other systems in which the La protein plays a role such as in cells infected with poliovirus, herpes simplex virus, and Rinderpest virus (Bachmann et al., 1989; Kurilla and Keene, 1983; Meerovitch et al., 1993; Raha et al., 2004). Therefore, our observations that the human and mosquito La protein interact with the 5′- and 3′-UTR of viral genome and the 3′-UTR (−) RNA as well as the human La protein interacts with NS3 and NS5 (García-Montalvo et al., 2004) suggested that the La protein would also be redistributed from the nucleus to the cytoplasm of DEN-infected cells. To examine this possibility, we analyzed the localization of La protein by confocal microscopy in uninfected and infected C6/36 cells, using mouse anti-mosquito La protein and rabbit anti-NS3 protein as primary antibodies and an anti-mouse conjugated with FITC and anti-rabbit conjugated with CY5 as secondary antibodies. In uninfected C6/36 cells, mainly a nuclear distribution of La protein (Fig. 5A) was seen by its colocalization with the propidium iodide stained nuclei. However, after 24 h of infection with DEN4 (Figs. 5B and C) and DEN2 (Figs. 5D and E), most of the cells showed mainly cytoplasmic distribution of La protein in punctate arrangement (Fig. 5B). This observation is more evident by enlargement of the images of infected cells (Figs. 5C and E). Moreover, in most of the cells that showed redistribution of La protein in cytoplasmic foci, NS3 protein was in some places colocalized with the La protein.

La protein inhibits DEN replication in vitro

To analyze the role of La protein in DEN replication, we used an *in vitro* RdRP assay. The assay was carried out using the subgenomic DEN2 viral RNA_{770nt} as the template (You and Padmanabhan, 1999) and the purified recombinant NS5

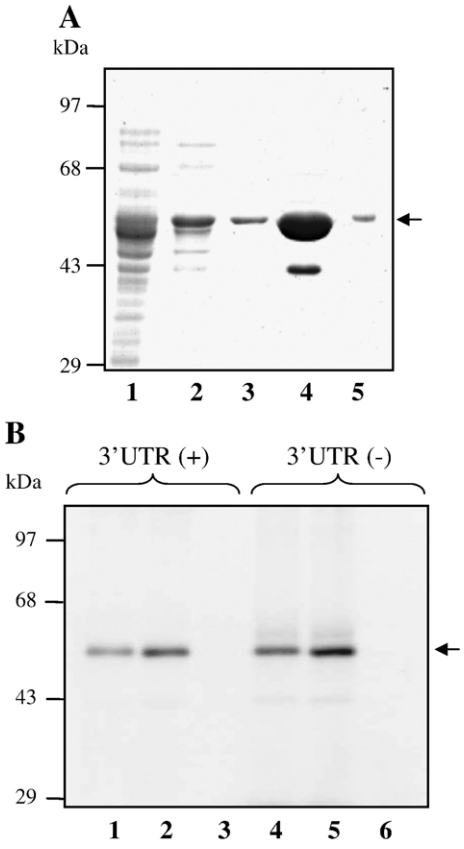

Fig. 4. Purification of mosquito La recombinant protein and UV-crosslinking assay with the 3′-UTRs of DEN4. (A) The purification procedure was performed using Ni-NTA agarose (Qiagen) column according to the manufacturer's instructions. The unbound fraction (lane 1), washes obtained in the presence of 10 and 20 mM of imidazole (lanes 2 and 3 respectively), elution fraction obtained with 250 mM of imidazole (lane 4) and the eluted mosquito La protein dialyzed and concentrated by Microcon columns (Millipore) (lane 5) were subjected to a SDS-10% PAGE and stained with Coomassie blue. (B) Labeled 3′-UTR (+) (lanes 1 to 3) and 3′-UTR (−) (lanes 4 to 6) were UV-crosslinked with 3 nM (lanes 1 and 4) and 6 nM (lanes 2 and 5) of recombinant mosquito La protein or 6 nM of recombinant GST protein (lanes 3 and 6). Proteins were separated by SDS-10% PAGE and detected by autoradiography. Molecular weight markers are indicated on the left.

(Ackermann and Padmanabhan, 2001) or DEN-infected C6/36 crude cytoplasmic extracts. The template RNA contains the 5′End$_{230nt}$ and complete 3′-UTR$_{451nt}$ of DEN2 genome. In this *in vitro* system, (−) strand RNA synthesis on subgenomic (+) RNA template generated two RNA products, a template-size RNA, which is the product of de novo synthesis of RNA by the viral replicase (denoted as 1×), and the other is the RNA product of elongation synthesis from the 3′-OH end of the template (denoted as 2×). In the presence of purified NS5, the two main products were produced, the first one the 2× product and the second one like an incomplete 2× product (Fig. 6A, lane 1). The same two main bands were observed when 6 nM of GST was added to the in vitro assay (Fig. 6A, lane 3). On the other hand, addition of the recombinant human La protein, obtained as a GST-La fusion protein, at the same concentration caused a complete inhibition of the RNA synthesis (Fig. 6A, lane 2). We chose to use human La protein because it was previously demonstrated to be functional in poliovirus translation in vitro (Shiroki et al., 1999).

RdRP assay was also performed using cytoplasmic extract from C6/36 infected with DEN4 as a source of NS5. Under this condition, RNA synthesis on a subgenomic RNA (+) template generated two bands, a template size band (1×) and the 2× size band (Fig. 6C, lane 1). The same products were observed in the presence of 6 nM of GST (Fig. 6C, lane 5), but in the presence of 3, 6 and 9 nM of recombinant La protein (Fig. 6C, lanes 2–4 respectively) an important dose dependent reduction in the amount of 1× and 2× products was detected. These results suggest that La protein plays a regulatory role in the synthesis of the (−) strand RNA.

In order to evaluate the effect of La protein in the positive strand RNA synthesis, the RdRP assay was also performed using the (−) strand subgenomic RNA as the template. In the presence of the recombinant NS5 protein, two main products, the 2× product and a very low amount the 1× product, were generated (Fig. 6B, lane 1). A similar pattern of RNA synthesis was obtained in the presence of 6 nM GST (Fig. 6B, lane 3). However, when the RdRP assay was performed in the presence of 6 nM of human La protein, the synthesis of the 2× product was completely inhibited, but the synthesis of the 1× product was still observed (Fig. 6B, lane 2).

Using the DEN4 infected C6/36 cytoplasmic extract as the source of replicase (Fig. 6D, lane 1) or in the presence of 6 nM of GST as control (Fig. 6D, lane 5), the de novo product 1× was observed as the main product with a much reduced amount of the 2× form. However, in the presence of 3, 6 or 9 nM of La protein, a dose-dependent inhibition in 1× and 2× product synthesis was also observed (Fig. 6D, lanes 2, 3 and 4 respectively). These results from *in vitro* viral replicase assays, taken together, suggest that La protein has inhibitory effects on (+) and (−) strand RNA synthesis in vitro.

Discussion

There are a number of reports regarding participation of host factors in the life cycle of (+) strand RNA viruses and specific binding of these factors to the 5′- and 3′-UTR of viral genomes. For example, the polypyrimidine tract-binding (PTB) protein and the heterogeneous nuclear ribonucleoprotein L (hnRNP L) bind to the HCV IRES and are presumably involved in viral translation (Ali and Siddiqui, 1995; Hahm et al., 1998). Human PTB protein and human La autoantigen interact with the 3′-UTR of genomic DEN4 (De Nova-Ocampo et al., 2002) and HCV RNA (Domitrovich et al., 2005). It is possible that common cellular components required for viral growth are present in different types of susceptible cells as well as cell-type-specific factors. Therefore, the presence or absence of specific factors required for viral replication can determine virus tropism, virulence or attenuation as has been described for other viruses (Gutierrez et al., 1997; Guest et al., 2004). Other proteins such as human calreticulin and protein disulfide isomerase interact *in vitro* with the 3′-UTR of the negative strand DEN4 RNA, the template on which the positive strand RNA is synthesized (Yocupicio-Monroy et al., 2003).

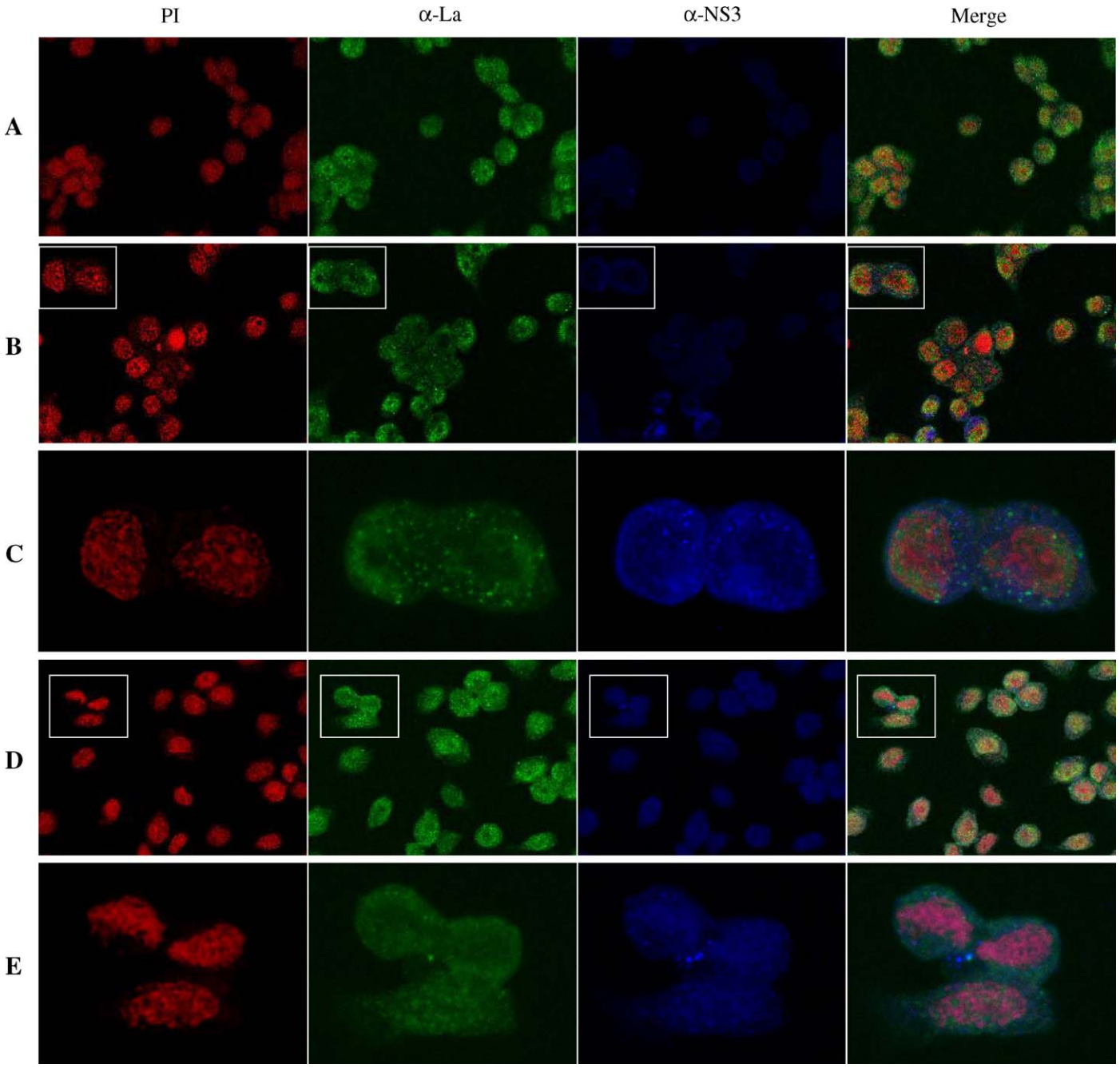

Fig. 5. Immunolocalization of mosquito La protein in C6/36 cells. C6/36 cells were uninfected (A), infected with DEN4 (MOI 0.5) (B and C) or infected with DEN2 (MOI 0.5) (D and F). After 24 h of infection, cells were fixed with paraformaldehyde and incubated with mouse anti-mosquito La antibody and rabbit anti-NS3 antibody, then incubated with anti-mouse conjugated with FITC and anti-rabbit conjugated with CY5 as secondary antibodies. Finally, the nuclei were stained with propidium iodide (PI), mounted and analyzed in a Leica TCS SP2 confocal microscope.

Calreticulin also interacts with a structure located in the genome of another positive strand RNA virus such as rubella virus (Singh et al., 1994).

DEN can be grown in human, primate and mosquito cells. Most of the host factors that have been found to interact with the DEN RNA sequences were isolated from human cells with the exception of the translation elongation factor-1 alpha (EF-1α) which was identified in the mosquito cell line, C6/36 (De Nova-Ocampo et al., 2002).

DEN infection is transmitted to humans by the bite of an infected mosquito. A productive infection in mosquito requires DEN to replicate efficiently in mosquito cells, making our study of the cellular and viral proteins required for translation and replication of DEN virus in mosquito cells relevant. If DEN virus like other (+) strand RNA viruses uses cellular proteins to regulate its translation and replication, the proteins that mediate translation and/or replication in human or primate cells could also play a similar function in mosquito cells; however, it is also

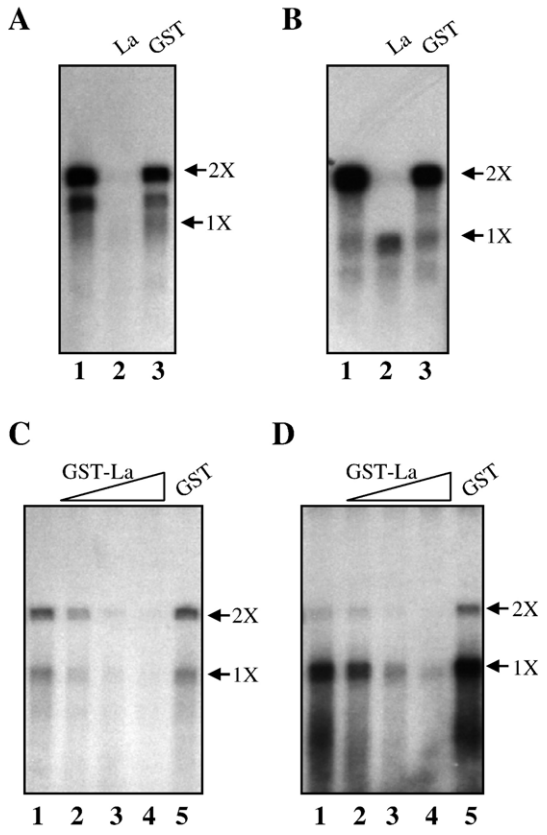

Fig. 6. RdRp assay with the subgenomic RNA and La protein. The NS5 DEN2 recombinant protein (A and B) or 5 μg of crude DEN 4 infected C6/36 cell extracts (C and D) were incubated with 1 μg of DEN2 subgenomic$_{770\ nt}$ of positive (A and C) and negative (B and D) polarity in the presence of UTP, ATP, GTP and ^{32}P labeled CTP. Recombinant La-GST protein was added to the reaction mix at 3 (C and D, lane 2), 6 (A and B, lane 2; C and D, lane 3) or 9 nM (C and D, lane 4) of recombinant human La-GST protein as well as 9 nM of recombinant GST protein (A and B, lane 3; C and D, lane 5). After incubation for 1.5 h, the synthesized RNAs were purified and separated by formaldehyde–1.5% agarose gel. The bands were detected by autoradiography. Arrows indicate migration of 1× and 2× RNA products.

possible that other cellular proteins in addition may be needed for those processes in different hosts. Since the cellular proteins required for DEN replicative cycle are poorly studied in mosquito cells, and due to the importance of this host in DEN transmission, the study of cellular proteins and specifically of mosquito La protein in DEN replication was investigated.

In the present report, we demonstrate that either the endogenous or the recombinant mosquito La protein is able to interact with the 3′-UTR of the (+) and (−) strand RNA from DEN4. To understand its role in the virus life cycle, we initially sought to examine its localization in infected cells by confocal microscopy. The results indicate that the La protein is present mainly in the nuclei of uninfected cells whereas it is re-distributed in the cytoplasm of DEN-infected cells. Moreover, La protein is colocalized with the viral protein NS3 in some foci, suggesting that this protein could be playing a role during viral replication. This result is consistent with our previous report that the human La protein coimmunoprecipitated with NS3 and NS5 (García-Montalvo et al., 2004). Moreover, this study is the first to demonstrate redistribution of La protein during DEN infection.

Our initial approach to determine the role of La protein in DEN replication was using an exogenous subgenomic RNA-dependent *in vitro* replication assay previously described (You and Padmanabhan, 1999; Ackermann and Padmanabhan, 2001). Using this system, we found that La protein completely inhibited the (−) strand RNA synthesis from (+) strand RNA templates using either recombinant NS5 protein or cytoplasmic cell extracts from DEN4-infected C6/36 cells. However, La protein inhibition of (+) strand synthesis from the subgenomic RNA of (−) strand polarity showed subtle differences from those of (−) strand synthesis from the (+) strand RNA template. At each concentration of La protein tested, RNA synthesis by 3′-end elongation of the (−) strand template (2× form) was preferentially inhibited compared to the de novo product (1× form) although both forms were inhibited in a dose-dependent manner. This observation suggests that either La protein alone or with one or more proteins present in the cellular extract could interact with the 3′-UTR of the (−) strand RNA preferentially inhibiting the 3′-end copy back mechanism. The significance of this finding is unknown at present and requires a more detailed study.

The inhibitory effect of La protein in viral RNA synthesis from both (+) and (−) strand RNA templates may be a consequence of its ability to interact with both 3′-UTRs such that this interaction may interfere with genome cyclization (De Nova-Ocampo et al., 2002; García-Montalvo et al., 2004; Yocupicio-Monroy et al., 2003). An alternate possibility is that La protein's ability to interact with NS3 and NS5 might interfere with the function of these components in viral replicase activity. In this regard, La protein may serve as a component of translation-to-replication switch mechanism. If we consider the sequence of events after (+) strand RNA virus entry, the viral genome is first released in the cytoplasm of infected cells. Then, the viral RNA is translated and when adequate amounts of viral proteins have been synthesized, it is replicated. Since translation and replication occur over the same RNA molecule but in opposite directions, one of the processes has to be blocked to allow the other to start. For DEN, several lines of evidence suggest that early viral translation does not require RNA genome cyclization (Alvarez et al., 2005b), although replication does depend on this requirement (Alvarez et al., 2005a; 2005b). Then, it is possible that specific conformational changes induced in the RNA and/or interaction of both ends of the viral genome with cellular proteins such as La protein (De Nova-Ocampo et al., 2002; García-Montalvo et al., 2004) could serve to block RNA cyclization. When adequate amounts of viral proteins accumulate in the endoplasmic reticulum, NS3 and NS5 could be interacting with La protein, releasing both ends of the viral genome, allowing viral cyclization and replication.

While the NS3 and NS5 are active in synthesis of (−) strand RNA from the (+) strand RNA template, they release the La protein, which is now available to interact with the 3′-UTR of the nascent (−) strand RNA. In this context, the La protein may be involved in localized melting of the double-stranded RNA

replicative form prior to initiation of (+) strand RNA synthesis. In this regard, the RNA helicase activity of La protein in conjunction with that of NS3 (Yon et al., 2005) may play a role (Hühn et al., 1997). A similar role for PTB protein in HCV replication has been proposed (Domitrovich et al., 2005). PTB interacts with both ends of HCV genomic RNA, interacts with NS3 and NS5B, and it is redistributed to the cytoplasm.

Recently, it was reported that La protein interacts with the nonstructural protein NS5A of HCV (a member of flavivirus family) (Houshmand and Bergqvist, 2003). Although the function of the NS5A protein has not been completely understood, it interacts with other proteins such as NS3, NS4B and NS5B that are likely to be important for assembly of a functional replication complex (Lin et al., 1997). Moreover, silencing of the La protein inhibited HCV translation and replication, supporting the idea that this protein plays a role in viral replicative cycle (Domitrovich et al., 2005).

The La protein may be involved in the life cycle of some viruses, such as the vesicular stomatitis virus (Wilusz et al., 1983), human parainfluenza virus (De et al., 1996), HCV (Ali and Siddiqui, 1997; Domitrovich et al., 2005; Spångberg et al., 2001), and rubella virus (Pogue et al., 1996). The La protein could play a role in different viral processes, including translation (Ali and Siddiqui, 1997; Domitrovich et al., 2005; Meerovitch et al., 1993), replication (Domitrovich et al., 2005; Pardigon and Strauss, 1996), and stabilization of viral RNA (Spångberg et al., 2001).

Although the cumulative results strongly point out that La protein plays an important role in DEN replication, its function in translation, in switching translation to replication or viral RNA stability remains to be explored. Further experiments directed to analyze both aspects are currently in progress in our laboratory.

Materials and methods

Cells and virus

Monolayers of C6/36 cells (from *Aedes albopictus*), kindly provided by Dr. Goro Kuno (CDC, Puerto Rico), were grown at 34 °C in Joklik modified minimal essential medium (MEM) (GIBCO) supplemented with nonessential amino acids, vitamins, 10% of fetal calf serum (Hyclone), penicillin and streptomycin.

DEN4 strain, H-241, generously provided by Dr. David Vaughn (Walter Reed Army Institute of Research, Washington, DC) and DEN2 strain 16681, generously provided by Richard Kinney from the CDC, Fort Collins, CO were propagated in suckling mice brain and in C6/36 cells as described previously (Gould and Clegg, 1991).

Construction of DNA templates for in vitro transcription

The DNA templates for *in vitro* transcription of the complete 3′-UTR (−) from DEN4 (from nt 101 to 1), del-62 [deletion from nt 62 to 68 within the 3′-UTR (−)] and del-96 [deletion from nt 96 to 101 within the 3′-UTR (−)] were produced as previously described (Yocupicio-Monroy et al., 2003). The DNA templates for in vitro transcription of the complete 3′-UTR (+) from DEN4 were prepared as described previously (De Nova-Ocampo et al., 2002).

Transcription of PCR products

PCR products were used to synthesize ^{32}P-labeled RNA transcripts by *in vitro* transcription in the presence of (α^{32}P) UTP (NEN) with T7 RNA polymerase following the conditions described by the manufacturer (New England Biolabs). Transcription reactions were treated with RNase-free DNase (1U) (Roche) at 37 °C for 20 min and later precipitated with ethanol/sodium acetate 3 M pH 5.8. The purified RNAs were resuspended into DEPC treated water (Sigma).

Preparation of S10 extracts

C6/36 cells uninfected and infected (48 h) with DEN4 were scraped in PBS and centrifuged at 1000 rpm. Cells were resuspended in 5 volumes of buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) and centrifuged at 3000 rpm at 4 °C for 5 min. Finally, cells were resuspended in 1 volume of buffer A and proteases inhibitor cocktail (Roche) and lysed with 28 strokes in a Dounce homogenizer. The homogenate was centrifuged at 10,000 rpm for 30 min in a Sorvall GSA rotor and the supernatant (S10 extract) was divided into aliquots and stored at −70 °C. The amount of protein was determined using the Bradford assay kit (Pierce).

Electrophoretic mobility shift assay

3 μg of S10 extract from DEN4 infected and uninfected C6/36 cells were preincubated with 4 μg of yeast tRNA (Sigma) in a buffer containing 10 mM HEPES pH 7.4, 0.02 mM DTT, 8 mM $MgCl_2$, 0.1 mM EDTA, 4 mM spermidine, 0.15 mM ATP, 1 mM GTP and 10% glycerol in a final volume of 10 μl at 4 °C for 15 min. Labeled RNA (1×10^6 cpm) was added and the mixture was incubated for 15 min at 4 °C for complex formation. Binding reactions were incubated with RNase T1 (20 U) for 20 min at room temperature and loaded gently onto a 6% polyacrylamide gel (acrylamide/*bis*-acrylamide 80:1) prepared in 0.5× TBE and electrophoresed at 20 mA for 3 h. Gels were dried and subjected to autoradiography.

For competition experiments, unlabeled RNAs were included in the preincubation reaction. For supershift assays, antibodies were added in the preincubation reaction.

UV-induced crosslinking

50 μg of S10 extract or different concentrations of recombinant La protein was incubated with 10 mM HEPES, 3 mM $MgCl_2$, 40 mM KCl, and 5% glycerol for 15 min 4 °C. Subsequently, 2×10^6 cpm of each of the ^{32}P-labeled RNAs was added to the mixture and incubated for additional 15 min at 4 °C. The reaction mixture was then irradiated with 254 nm UV light (UV Ultra-Lum lamp) at 3 cm for 15 min on ice and treated with RNAse A and T1 for 15 min at 37 °C. Samples were analyzed by SDS-10% PAGE. Gels were fixed, dried and autoradiographed.

Immunoprecipitation with anti-La antibodies

After UV crosslinking, the samples were incubated with 10 μl of protein G–agarose beads (Gibco) for 2 h at 4 °C and centrifuged at 12,000 rpm for 5 min. Supernatants were incubated with monoclonal anti-La antibody (kindly provided by N. Sonenberg, McGill University, Montreal, Quebec, Canada) in the presence of protein G–agarose beads saturated with bovine serum albumin overnight at 4 °C. Unbound materials were removed by washing five times with NETS buffer (50 mM Tris–HCl pH 7.4, 5 mM EDTA, 1 mM DTT, 100 mM NaCl, 0.05% Nonident P40). Bound proteins were analyzed by SDS-10% PAGE followed by autoradiography. Parallel analysis was performed using an unrelated anti-actin monoclonal antibody (kindly provided by Manuel Hernández, CINVESTAV-IPN, México) as control.

Western blot assay

Samples were fractionated through SDS-10% PAGE and transferred to nitrocellulose membranes by using a semidry Transblot apparatus at 16 V for 30 min with a transfer buffer (0.025 M Tris–HCl pH 9.5, 0.019 M glycine, 20% [vol/vol] methanol). Membranes were blocked for 2 h at room temperature in skimmed milk and then washed three times with washing buffer (PBS-Triton 0.5% [wt/vol]). The anti-His rabbit polyclonal antibody (Santa Cruz Biotechnologies) was incubated with the membrane overnight at 4 °C. The secondary antibody, the anti-rabbit immunoglobulin G conjugated to peroxidase (Amersham), diluted 1:30,000 was incubated at room temperature for 1 h. The assay was developed using supersignal west femto (Pierce) following the manufacture's instructions.

Construction of pLaMos plasmid

The coding region of mosquito La protein [NCBI accession number *S80954*], flanked by Sal I restriction sites, was amplified from C6/36 RNA extract as follows. First, a reverse primer (5′gtcgacatgaccgaagtcgaagc3′) and superscript II RT (Invitrogen) was used for the reverse transcriptase reaction according to the manufacturer's protocol. Subsequently, the PCR step was performed with Pfu polymerase (Invitrogen) and the forward primer (5′gtcgacttactctcctcgtccgc3′) in a Perkin Elmer Cetus DNA thermal cycler 480 (30 cycles at 94 °C for 30 s, 55 °C for 1 min and 68 °C for 1.5 min). The amplified product was cloned in PCR II TOPO TA cloning vector (Invitrogen) and after digestion with the restriction enzyme Sal I, the released fragment was cloned into the pProEX-HTc expression vector (Invitrogen) to yield the pLaMos plasmid (5943 bp) which consists of the ORF for mosquito La protein fused in-frame to a 6-His tag. The sequence of the mosquito La protein gene was verified and was found to correspond to the expected sequence. *E. coli* cells (DH5α) transformed with this plasmid were grown up to an O.D. of 0.6 and were induced at 37 °C for 5 h in the presence of 0.5 mM of isopropyl-thio-galactoside (IPTG). The preparation of bacterial extract and the purification on a column of Ni-NTA agarose (Qiagen) were performed according to the manufacturer's instructions. The recombinant mosquito La protein was dialyzed and concentrated using microcon concentrator columns (Millipore).

Immunofluorescence

Cells grown on coverslides were infected with DEN2 or DEN4 at a MOI of 0.5. After 24 h of infection, cells were washed with PBS and fixed with 4% of paraformaldehyde for 1 h and then washed and permeabilized with acetone for 2 min on ice. Then, cells were washed and incubated with the blocking solution (PBS–10% fetal bovine serum) for 1 h at room temperature. After this treatment, cells were washed and incubated overnight at 4 °C with the mouse anti-mosquito La antibody diluted 1:50 or rabbit anti-NS3 antibody 1:30. Cells were washed and incubated with FITC labeled anti-mouse IgG 1:80 (Zymed) and CY5 labeled anti-rabbit IgG 1:60 (Zymed) for 1 h at room temperature, then after a washing step, nuclei were stained with propidium iodide solution (Sigma). Slides were mounted with Vectashield (Vector) and analyzed using a Leica TCS SP2 confocal microscope.

RdRp assay with recombinant La protein

The reaction mixtures contained 50 mM HEPES pH 7.3, 10 mM KCl, 10 mM $MgCl_2$, template RNA (1 μg), 500 μM each ATP, GTP UTP, 10 μM unlabeled CTP, 10 μCi of ^{32}P CTP, crude cytoplasmic DEN4 infected C6/36 cell extracts and GST-human La protein (De Nova-Ocampo et al., 2002) or recombinant GST as indicated. The reaction was carried out by incubation at 30° for 90 min and terminated by acid phenol/chloroform extraction, followed by ethanol precipitation after the addition of yeast tRNA (5 μg) as a carrier and resuspended in DEPC-treated water. This RNA was passed through P-30 column (Bio-Rad) and was precipitated with ethanol. RNA was analyzed by formaldehyde–agarose gel electrophoresis and visualized by autoradiography.

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